Agar Diffusion Procedures for Susceptibility Testing of Malasseziapachydermatis: Evaluation of Mueller-Hinton Agar Plus 2 % Glucose and 0.5 µg/ml Methylene Blue as the Test Medium

Agar diffusion procedures for susceptibility testing of *Malassezia pachydermatis*: evaluation of Muller-Hinton agar plus 2% glucose and 0.5 µg/ml methylene blue as the test medium

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Abstract

Aim of this study was to verify whether Mueller-Hinton agar supplemented with 2% glucose and methylene blue (MH-GM), which is used for disk diffusion susceptibility testing of *Candida* species by the Clinical and Laboratory Standards Institute, is suitable for testing *Malassezia pachydermatis*. A variant of the disk diffusion procedure utilizing a 9-mm tablet was used to test 31 isolates against clotrimazole and miconazole using MH-GM as test medium. The MH-GM agar optimally supported the growth of all *M. pachydermatis* isolates provided that the yeast inoculum was prepared with a lipid source (Tween 40 and 80). Zone edges were frequently definite and clear, facilitating the measurement of zone size and minimizing subjectivity. The inhibition zones correlated with MIC values obtained in a broth dilution assay.

The agar diffusion method with MH-GM as the test medium appears as a suitable screening procedure for testing the susceptibility of *M. pachydermatis* to CTZ and MCZ in clinical laboratories. This test format may allow processing a large number of isolates in epidemiological studies. This may in turn facilitate clarifying to what extent the problem "drug resistance" accounts for cases of treatment failure in dogs with *Malassezia* otitis and dermatitis.

Keywords

Malassezia pachydermatis; Susceptibility testing; Agar; Mueller-Hinton; dog

1. Introduction

Malassezia pachydermatis, the sole lipophilic, but not lipid-dependent, species of *Malassezia*, is a normal inhabitant of canine skin and ear canals. Favorable growth conditions in the local skin environment allow excessive multiplication of this organism, which may then function as an opportunistic secondary pathogen in dermatitis and otitis externa [1].

Canine *Malassezia* dermatitis and otitis have been reported to be responsive to a variety of antifungal medications, including azole derivatives, nystatin, and terbinafine [2]. However, treatment failure is commonly reported in clinical practice, especially for otitis, and whether failure is due to incorrect management of conditions underlying the yeast overgrowth or antifungal resistance of the yeast is unclear. Some studies support the latter possibility, reporting isolate resistance to commonly used drugs, such as thiabendazole, clotrimazole, miconazole, and itraconazole[3–6]. However, other studies have reported substantial activity for these drugs [7–10]. Such discrepancies are due to the fact that *in vitro* susceptibility tests are still largely unstandardized with regard to *M. pachydermatis*. Thus, this issue would benefit from the development of a testing method capable of standardization and routine application in testing a large number of *Malassezia* isolates.

A disk diffusion procedure has been standardized for *Candida* species by the Clinical and Laboratory Standards Institute (CLSI, document M44-A2) [11]. The precision of this method can be compared to that of minimum inhibitory concentration (MIC, µg/ml) determinations using the reference broth dilution-based assay for testing the antifungal susceptibility of yeasts (CLSI document M27-A3)[12,13].Compared with this reference procedure, the disk diffusion method has been shown to be more user-friendly and practical [13]. Most studies of the antifungal susceptibility of *M. pachydermatis* have been based on broth dilution procedures. As mentioned, these studies have not yet led to establishing a reference procedure, but they at least allowed a definition of technical parameters that may be suitable for this type of assay [7,10,14–24]. On the other hand, the value of agar diffusion procedures has been investigated less comprehensively, with a minority of

studies employing such methodology [4,5,7,9,10]. In addition, no study has ever demonstrated whether inhibition zones correlate with MIC values as has been done with *Candida* spp. and other yeast species[13,26]. Moreover, the growth media employed for agar diffusion testing of *M. pachydermatis* (Sabouraud dextrose agar - SDA [6,9], SDA with 1% Tween 80 and 1.5% yeast extract [4], Casitone agar [3,8]), were not evaluated with regard to their fitness in producing clear and easy to interpret inhibition zone edges. Yet, this issue has an obvious impact on the accuracy of reading and, consequently, the repeatability of results [26,27]. The CLSI reference assay (document M44-A2) uses Mueller-Hinton (MH) agar supplemented with 2% glucose (G) and methylene blue (M), since it produces clear inhibition zone edges and less intrazonal growth, enabling easy interpretation of inhibition zone diameters [11]. In a recent publication, MH-GM agar has been used for the first time also for testing *M. pachydermatis*, in particular towards ketoconazole, itraconazole, nystatine, terbinafine and 5-fluorocytosine [25]. The aim of the present study was to contribute to ascertain the suitability of this medium for susceptibility testing of *M. pachydermatis* using other two drugs of common use in the dog, namely clotrimazole and miconazole.

2. Materials and methods

Clotrimazole (CTZ) and miconazole (MCZ) were tested against 31 clinical isolates of *M. pachydermatis*, including a reference strain (*M. pachydermatis* CBS 1879) for which we previously found a range of MICs for both drugs [10]. We used a variant of the disk diffusion procedure that utilizes a 9-mm tablet (NeoSensitabs, 10 µg diffusible amount; Rosco, Taastrup, Denmark) and has been favorably investigated for testing *Candida* spp. [28] The method was performed according to the manufacturer's instructions and M44-A2 guidelines [11]. Uniform yeast suspensions were prepared in the culture medium used for the broth dilution assay (Christensen's urea broth with Tween 80 and Tween 40 as the lipid source) and vortexed with glass beads to avoid yeast cell clustering. Inoculum suspensions were adjusted by spectrophotometry to approximately $1–5 \times 10^7$ colony-forming units (cfu)/ml. Lower-concentration inoculums, such as that recommended by CLSI for *Candida* spp. (0.5 McFarland standard, corresponding to 1×10^6 - 5×10^6 cfu/ml) [11] and

suspensions without a lipid source, e.g prepared in sterile saline, were shown to not provide adequate confluent growth of *Malassezia* on agar plates in preliminary experiments (data not shown).

3. MH agar (Biolife Italiana S.r.l, Milan, Italy) was solidified after the addition of 2% glucose and 5 mg methylene blue/ml (Sigma Aldrich, Milan, Italy). GM-MH agar plates (150-mm plates) were inoculated simultaneously in three directions using a nontoxic cotton swab dipped in the inoculum suspension. After the plates were dried for 15 min, CTZ and MCZ tablets were applied to the inoculated agar with a pair of forceps. The plates were incubated in ambient air at 37°C. The diameter of each zone of inhibition was determined as the area showing a sharp decline in the density of growth (CLSI, 2009). For the broth-dilution assay we used a modified CLSI M27-A3 microdilution method (CLSI, 2008), which was described previously [10]. Briefly, a 96-well microtiter plate containing progressive dilutions of the antifungals across rows (16-0.03 µg/ml) was inoculated with the yeast culture (1:100 dilution of the suspension used in the tablet diffusion method, final inoculum 1-5 × 10^5 cfu/ml). The test medium was Christensen's urea broth with Tween 80 and Tween 40 as the lipid source in accordance with Rincon et al. (2006) [15], who showed that this medium optimally supports the growth of *Malassezia* spp. and does not interfere with antifungal activity. Plates were incubated at 37°C and read 48 h after inoculation. Azole MICs were the lowest drug concentrations showing an optical density of ≤50% of the drug-free growth control as assessed by spectrophotometry (630 nm filter) [12]. Zones of inhibition were plotted against the MICs determined by the microdilution method and the least-squares method was used to calculate regression lines. The correlation between diameters and MICs was evaluated by the Spearman test. **Results**

The MH-GM agar optimally supported the growth of all *M. pachydermatis* isolates tested. Zone edges were frequently definite and clear. The methylene blue gave the agar plates a slight blue color and offered better contrast between the growth/no growth zone, facilitating the measurement of

zone size and minimizing subjectivity (Fig. 1). During the first 24 h, most strains grew too poorly to permit measurement of zone diameters, but they all grew sufficiently after 48 h. Zone diameters at 72 h and subsequent controls (up to 5 days) were unmodified. Therefore, we chose 48 h as the time of reading.

Table 1 presents the inhibition zone diameters corresponding to each MIC obtained in the broth-dilution assay. MIC diameters were generally wider around CTZ tablets, but the same tendency was noted for both drugs, namely increased MIC was accompanied by a progressive decrease in the diameter of the inhibition zone. This tendency was confirmed by regression statistics calculated from a scattergram plotted to compare the 48-h inhibition zones and the microdilution MICs for each of the 31 strains (Fig. 2). From the regression lines calculated, a two-fold concentration change in MICs was accompanied by about 6 (CTZ) and 3 mm (MCZ) change in the diameter of the zone of inhibition. Moreover, Spearman correlation test yielded a significant negative correlation between MIC values and diameters of inhibition both for CTZ (rho= - 0.59; $p< 0.05$) and MCZ (rho= - 0.55; $p< 0.05$). The mean intra-assay coefficient of variation was 5.1% and 4.4% for CTZ and MCZ, respectively, indicating good reproducibility of the results for both drugs.

4. Discussion

The present study was aimed to set up laboratory parameters adequate to obtain reliable indications on the *in vitro* susceptibility of *M. pachydermatis* to two azole drugs, CTZ and MCZ, using an agar diffusion procedure. In particular we focused on the growth medium, which is recognized to play a key role for this type of test [13,27,29]. The MH-GM medium employed is recommended by the CLSI for testing *Candida* species in disk diffusion assay [11]. Indeed, several multicenter studies have shown that this medium is superior to other agars because it is readily available, shows acceptable batch-to-batch reproducibility, and produces clear inhibition zone edges and less intrazonal growth, enabling easy interpretation of inhibition zone diameters [29]. Our findings support the validity of this agar also for susceptibility testing of *M. pachydermatis* with agar diffusion procedures, provided that the yeast inoculum is prepared with a lipid source (Tween 40

and Tween 80 in the present study). In particular, the production of clear and reproducible inhibition zones that correlate with MIC values suggest that the agar diffusion method with MH-GM agar can be used as a screening procedure for the susceptibility of *M. pachydermatis* to CTZ and MCZ in clinical laboratories. This test format, more simple and rapid than the broth microdilution procedure, may allow processing a higher number of isolates in epidemiological studies. This may in turn facilitate clarifying to what extent the problem "drug resistance" accounts for cases of treatment failure in dogs with *Malassezia* otitis and dermatitis that undergo antifungal treatment with CTZ and MCZ. The use of MH-GM rather than of SDA, employed in past studies of *M. pachydermatis* using the disk diffusion method [4,6,9] might be preferable. Indeed, SDA has been reported, even though in a different test format (agar dilution assay), to obfuscate the antifungal activity of some antifungal agents, such as 5-fluorocytosine, CTZ, and MCZ [30,31].

In a recent study, Yurayart *et al.* [25] employed a disk diffusion procedure with MH-GM agar as growth medium for testing *M. pachydermatis* against other azole drugs – ketoconazole and itraconazole – and drugs belonging to other chemical classes, such as nystatine, terbinafine and 5-fluorocytosine. Unfortunately, this study does not document clearly the performances of MH-GM medium. Moreover most results are reported in a way difficult to interpret. For example, authors say that "Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg/l methylene blue was used for disk diffusion method to allow growth of *M. pachydermatis* and clearly presented an inhibition zone, except for KTZ and ITZ" [25]. Also the correlation of inhibition zones with MIC values is not clear, given that in the diagrams presented in this publication [25] a very low MIC value on the x-axis (< 0,03) appears to correspond on the y-axis to a wide range of inhibition diameters (from about 20 to 80 mm for itraconazole; from 25 to 90 mm for ketoconazole). Reading of the text does not contribute clarifying this issue, since authors report that "the scatter diagrams and the statistical analysis using linear regression could not explain the correlation between the CLSI MICs and inhibition zone diameters due to a lack of diversity among the susceptibility levels of *M. pachydermatis*"[25].

In conclusion, the present study documents clearly for the first time the suitability of MH-GM agar for agar diffusion susceptibility testing of *M. pachydermatis*, at least as far as CTZ and MCZ are concerned. Further experiments will be necessary to confirm the suitability of this medium for testing the activity of other antifungal agents.

Conflict of interest statement

None to declare

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FIGURE LEGENDS

Fig. 1. MCZ disk diffusion assay for a *Malassezia pachydermatis* strain, performed on Mueller-Hinton agar supplemented with 2% glucose and methylene blue. A clear inhibition zone edge is visible.

Fig. 2. Zones of inhibition around 10-µg clotrimazole or miconazole tablets plotted against the MICs determined by the microdilution method for 31 isolates of *Malassezia pachydermatis.* The least-squares method was used to calculate regression lines ($y = 63.6 – 5.59x$, with $R^2 = 0.4$ for CTZ; $y = 43.2 – 3.03x$, with $R^2 = 0.52$ for MCZ)